



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>5</sup> : A61K 31/415	A1	(11) International Publication Number: WO 94/18967 (43) International Publication Date: 1 September 1994 (01.09.94)
<p>(21) International Application Number: PCT/US94/01740</p> <p>(22) International Filing Date: 18 February 1994 (18.02.94)</p> <p>(30) Priority Data: 08/018,840 18 February 1993 (18.02.93) US</p> <p>(71) Applicant: PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 124 Mount Auburn Street, Cambridge, MA 02138 (US).</p> <p>(72) Inventors: HALPERIN, Jose; 1443 Beacon Street, Brookline, MA 02146 (US). BRUGNARA, Carlo; 33 Aberdeene Street, Newton Highlands, MA 02161 (US).</p> <p>(74) Agent: GATES, Edward, R.; Wolf, Greenfield &amp; Sachs, 600 Atlantic Avenue, Boston, MA 02210 (US).</p>	<p>(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published With international search report.</p>	
<p>(54) Title: TREATMENTS FOR DISEASES CHARACTERIZED BY NEOVASCULARIZATION</p> <p>(57) Abstract</p> <p>The applicant has identified a particular class of imidazoles that inhibit angiogenesis. These imidazoles can be used to beneficially treat a variety of angiogenic conditions.</p> <p><b>BEST AVAILABLE COPY</b></p>		

32 + 1

ATTORNEY DOCKET NUMBER: 10177-191-999  
SERIAL NUMBER: 10/603,115  
REFERENCE: B140



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>5</sup> : A61K 31/415	A1	(11) International Publication Number: WO 94/18967 (43) International Publication Date: 1 September 1994 (01.09.94)
(21) International Application Number: PCT/US94/01740 (22) International Filing Date: 18 February 1994 (18.02.94) (30) Priority Data: 08/018,840 18 February 1993 (18.02.93) US (71) Applicant: PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 124 Mount Auburn Street, Cambridge, MA 02138 (US). (72) Inventors: HALPERIN, Jose; 1443 Beacon Street, Brookline, MA 02146 (US). BRUGNARA, Carlo; 33 Aberdeene Street, Newton Highlands, MA 02161 (US). (74) Agent: GATES, Edward, R.; Wolf, Greenfield & Sacks, 600 Atlantic Avenue, Boston, MA 02210 (US).	(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  Published With international search report.	
(54) Title: TREATMENTS FOR DISEASES CHARACTERIZED BY NEOVASCULARIZATION (57) Abstract  The applicant has identified a particular class of imidazoles that inhibit angiogenesis. These imidazoles can be used to beneficially treat a variety of angiogenic conditions.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

-1-

TREATMENTS FOR DISEASES CHARACTERIZED  
BY NEOVASCULARIZATION

Field of the Invention

The invention relates to diseases characterized by neovascularization and the use of imidazoles that inhibit the  $\text{Ca}^{++}$  activated potassium channel in arresting or inhibiting such neovascularization.

Background of the Invention

A normal artery typically is lined on its inner-side by a single layer of endothelial cells, the intima. The intima overlays the media, which contains only a single cell type, the vascular smooth muscle cell. The outer-most layer of the artery is the adventitia.

Neovascularization, or angiogenesis, is the growth and development of new arteries. It is critical to the normal development of the vascular system, including injury-repair. There are, however, conditions characterized by abnormal neovascularization, including diabetic retinopathy, neovascular glaucoma, rheumatoid arthritis, psoriasis and certain cancers. For example, diabetic retinopathy is a leading cause of blindness. There are two types of diabetic retinopathy, simple and proliferative. Proliferative retinopathy is characterized by neovascularization and scarring. About one-half of those patients with proliferative retinopathy progress to blindness within about five years.

Another example of abnormal neovascularization is that associated with solid tumors. It is now established that unrestricted growth of tumors is dependant upon angiogenesis, and that induction of angiogenesis by liberation of angiogenic factors can be an important step in carcinogenesis. For example, basic fibroblast growth factor (bFGF) is liberated by several cancer cells and plays a crucial role in cancer

-2-

angiogenesis. The demonstration that certain animal tumors regress when angiogenesis is inhibited has provided the most compelling evidence for the role of angiogenesis in tumor growth.

It would be desirable to identify antiangiogenesis agents useful in treating the foregoing diseases.

Imidazoles are synthetic antifungal agents that are used both topically and systemically. Indications for their use include ringworm, tinea versicolor and mucocutaneous candidiasis. These compounds are believed to act by inhibiting ergosterol synthesis in the fungal cell wall, and when given topically, may cause direct damage to the cytoplasmic membrane.

The fungi comprise five widely differing classes of primitive flora, and the variation in cell physiology and biochemistry are extreme. As a result, most antifungal agents have a very narrow spectrum of antifungal activity.

Various imidazoles have been suggested as treatments for prostate cancer. The only one known to the applicants to have been tested is ketoconazole. Ketoconazole is an antifungal agent that, in high doses, inhibits testicular and adrenal synthesis of steroid hormones, including testosterone. The ability of ketoconazole to block steroid synthesis has prompted its use in the treatment of advanced prostate carcinoma because prostate cancer cells are highly dependent on testosterone. The major sites of action appear to be in the inhibition of 17-20 desmolase, partial blockade of 17-hydroxylase and marked inhibition of 21- and/or 11-hydroxylase, all major enzymes of the androgenic hormone synthetic pathways.

In the recent past, newer methods of androgen ablation for the treatment of metastatic prostate carcinoma have been developed as alternatives to the standard forms of therapy: oral estrogens and surgical castration. Luteinizing hormone-release hormone (LHRH) analogs, potent inhibitors of testosterone production, have recently emerged as major

-3-

players in the long term treatment of advanced prostate cancer. In contrast, ketoconazole has been found to be excellent for short-term usage prior to bilateral orchiectomy and when prompt therapeutic response is needed but orchiectomy cannot be performed. In high doses, ketoconazole causes castrate levels of testosterone within 24 to 48 hours; therefore, it is extremely useful in the initial medical treatment of patients with metastatic prostate cancer who need a prompt therapeutic response. Thus, ketoconazole has been used as a hormonal adjuvant for prostate cancer treatment; it reduces plasma testosterone to castration levels. Ketoconazole, as will be described below, is not useful for inhibiting endothelial and vascular smooth muscle cell proliferation associated with neovascularization.

#### Summary of the Invention

The applicants have identified a new class of potent anti-angiogenesis agents. These agents comprise a particular class of imidazoles that inhibit endothelial and vascular smooth muscle cell proliferation. These imidazoles can be used to beneficially treat a variety of angiogenic conditions, as described below.

According to the invention, a method for treating an angiogenic condition is provided. An imidazole is administered to a subject in need of such treatment. The imidazole is an inhibitor of the  $\text{Ca}^{++}$  activated potassium channel of erythrocytes of the subject. It also is an inhibitor of endothelial and/or smooth muscle cell proliferation. Preferred imidazoles are clotrimazole, miconazole and econazole.

The treatment typically is for tissues or subjects that are otherwise free of indications for the preferred imidazoles. As such, the tissue or subject being treated preferably is substantially free of a fungal infection calling for the treatment of the subject with the imidazoles of the invention.

-4-

According to another aspect of the invention, a sustained release implant containing an imidazole as described above is provided. The implant is constructed and arranged for the long-term delivery of the imidazole when implanted in vivo. Still another aspect of the invention, is a cocktail of anti-cancer agents, including an imidazole as described above.

#### Brief Description of the Drawings

Figure 1 is a graph illustrating the ability of clotrimazole to inhibit cell proliferation in vascular smooth muscle cells, and the ability to reverse the effects of clotrimazole treatment.

Figure 2 is a graph showing that clotrimazole inhibits DNA synthesis in a dose-dependent fashion.

Figure 3 is a graph comparing the effect upon cell proliferation of a variety of drugs.

Figure 4 is a graph comparing the effect upon the  $Ca^{++}$  activated potassium channel of the same drugs tested in connection with Figure 3.

Figure 5 is a graph illustrating the inhibitory effect that clotrimazole has upon complement-induced release of mitogenic activity from endothelial cells.

#### Detailed Description of the Preferred Embodiments

The invention is used in connection with treating an angiogenic condition. As used herein, an angiogenic condition means a disease or undesirable medical condition having a pathology including neovascularization. Such diseases or conditions include diabetic retinopathy, psoriasis, neovascular glaucoma, rheumatoid arthritis, cancers that are solid tumors and cancers or tumors otherwise associated with neovascularization such as hemangioendotheliomas, hemangiomas and Kaposi's sarcoma.

-5-

Proliferation of endothelial and vascular smooth muscle cells is the main feature of neovascularization. The invention is useful in inhibiting such proliferation and, therefore, inhibiting or arresting altogether the progression of the angiogenic condition which depends in whole or in part upon such neovascularization. The invention is particularly useful when that condition has as an additional element endothelial or vascular smooth muscle cell proliferation that is not necessarily associated with the unwanted neovascularization. For example, psoriasis may additionally involve endothelial cell proliferation that is independent of the endothelial cell proliferation associated with neovascularization. Likewise, a solid tumor which requires neovascularization for continued growth also may be a tumor of endothelial or vascular smooth muscle cells. In this case, the tumor cells themselves are inhibited from growing in the presence of the imidazoles used in the invention.

The invention is used in connection with treating subjects having, suspected of having, developing or suspected of developing such conditions. A subject as used herein means humans, primates, horses, cows, pigs, sheep, goats, dogs, cats and rodents.

The compounds useful in the present invention are imidazoles that inhibit the  $\text{Ca}^{++}$  activated potassium channel. Such imidazoles are either known to those of ordinary skill in the art or can be identified without undue experimentation using established tests routinely employed by those of ordinary skill in the art. One such test involves human erythrocytes and is described in the examples, below. When using this test, it is desirable to select imidazoles that are inhibitory to an extent of at least about 75%.

The imidazoles of the invention also inhibit endothelial and/or vascular smooth muscle cell proliferation. Inhibition of such proliferation may be tested without undue experimentation using established tests routinely employed by those of ordinary skill in the art (See examples, below.)



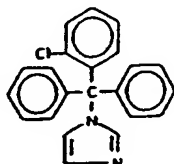
The imidazoles used in this invention preferably are inhibitory of endothelial and/or vascular smooth muscle cell proliferation in such tests to an extent of at least about 75%.

It was not expected that inhibitors of the  $\text{Ca}^{++}$  activated potassium channel would inhibit endothelial or vascular smooth muscle cell proliferation. Other specific inhibitors of the  $\text{Ca}^{++}$  activated potassium channel (such as charybdotoxin, kaliotoxin and iberiotoxin) do not inhibit proliferation of endothelial or vascular smooth muscle cells. Moreover, inhibitors of other transport systems that are activated by mitogens, such as ouabain (highly specific inhibitor of the Na/K pump) and amiloride (inhibitor of Na/H exchange) do not inhibit cell proliferation. Thus, the results obtained by the applicants are surprising.

Without limiting the invention to the use of the specific compounds listed, the following is a list of preferred compounds and well-characterized salts thereof useful in the methods of the invention.

#### Clotrimazole

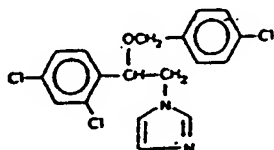
1*H*-Imidazole, 1-[(2-chlorophenyl)diphenylmethyl]-, Lotrimin (Schering); Mycelex (Miles)



1-(*o*-Chloro- $\alpha,\alpha$ -diphenylbenzyl)imidazole [23593-75-1]  
 $\text{C}_{22}\text{H}_{17}\text{ClN}_2$  (344.84).

#### Econazole

1*H*-Imidazole, ( $\pm$ )-1-[2-[(4-chlorophenyl)methoxy]-2-(2,4-dichlorophenyl)ethyl]-, mononitrate, Ecostatin (Squibb)



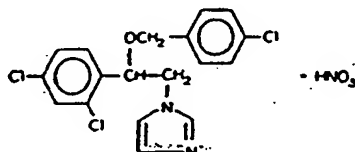
( $\pm$ )-1-[2,4-Dichloro- $\beta$ -(*p*-chlorobenzyl)oxy]phenethyl]imidazole mononitrate [68797-31-9]  $\text{C}_{18}\text{H}_{15}\text{Cl}_3\text{N}_2\text{O}$ .

**SUBSTITUTE SHEET**

-7-

**Econazole Nitrate**

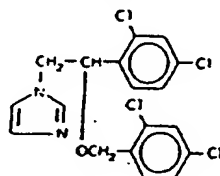
1*H*-Imidazole, (±)-1-[2-[(4-chlorophenyl)methoxy]-2-(2,4-dichlorophenyl)ethyl]-, mononitrate, Ecostatin (*Squibb*)



(±)-1-[2,4-Dichloro-β-[(*p*-chlorobenzyl)oxy]phenethyl]imidazole mononitrate [68797-31-9] C<sub>18</sub>H<sub>15</sub>Cl<sub>3</sub>N<sub>2</sub>O.HNO<sub>3</sub> (440.70).

**Miconazole**

1*H*-Imidazole, 1-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl)methoxy]ethyl]-, Monistat (*Janssen*)



1-[2,4-Dichloro-β-[(2,4-dichlorobenzyl)oxy]phenethyl]imidazole [22916-47-8] C<sub>18</sub>H<sub>14</sub>Cl<sub>4</sub>N<sub>2</sub>O (416.12).

**Miconazole Nitrate**

Monistat (*Ortho*)

[22832-87-7] C<sub>18</sub>H<sub>14</sub>Cl<sub>4</sub>N<sub>2</sub>O.HNO<sub>3</sub> (479.15).

The above imidazoles and salts thereof are well recognized, pharmacologically characterized, and licensed for use by the FDA today either as antimycotic agents or antiprotozoal agents. As such, established and empirically documented parameters regarding their limited toxicity and their useful dosages are well described in the scientific and medical literature.

The imidazoles used in the methods of the invention may be administered per se (neat) or in the form of a pharmaceutically acceptable salt. When used in medicine, the salts should be both pharmacologically and pharmaceutically

**SUBSTITUTE SHEET**

-8-

acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare the free active compound or pharmaceutically acceptable salts thereof. Pharmacologically and pharmaceutically acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulphuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluenesulfonic, tartaric, citric, methanesulphonic, formic, malonic, succinic, naphthalene-2-sulphonic, and benzenesulphonic. Also, pharmaceutically acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group. Thus, the present invention involves the use of pharmaceutical formulations which comprise certain imidazoles together with one or more pharmaceutically acceptable carriers and optionally other therapeutic ingredients. The carrier(s) and other ingredients of course must be pharmaceutically acceptable.

Analogous of the foregoing compounds that act as functional equivalents also are intended to be embraced as equivalents and within the scope of the invention.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular drug selected, the particular condition being treated and the dosage required for therapeutic efficacy. The methods of this invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces therapeutic levels of the imidazoles of the invention without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, topical, nasal, transdermal or parenteral (e.g. subcutaneous, intramuscular and intravenous) routes. Formulations for oral administration include discrete units such as capsules, tablets, lozenges and the

-9-

like. Other routes include intrathecal administration directly into spinal fluid and direct introduction onto, in the vicinity of, or within the target cells.

The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing the active imidazole into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the imidazole into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets, tablets or lozenges, each containing a predetermined amount of the imidazole, in liposomes or as a suspension in an aqueous liquor or non-aqueous liquid such as a syrup, an elixir, or an emulsion.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the imidazole, which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using those suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example as a solution in polyethylene glycol and lactic acid. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectibles.

-10-

Other delivery systems can include sustained release delivery systems. Preferred sustained release delivery systems are those which can provide for release of the imidazoles of the invention in sustained release pellets or capsules. Many types of sustained release delivery systems are available. These include, but are not limited to: (a) erosional systems in which the imidazole is contained in a form within a matrix, found in U.S. Patent Nos. 4,452,775 (Kent), 4,667,014 (Nestor et al.); and 4,748,024 and 5,239,660 (Leonard) and (b) diffusional systems in which an active component permeates at a controlled rate through a polymer, found in U.S. Patent Nos. 3,832,252 (Higuchi et al.) and 3,854,480 (Zaffaroni). In addition, a pump-based hardware delivery system can be used, some of which are adapted for implantation.

Use of a long-term sustained release implant may be particularly suitable for treating solid tumors. "Long term" release, as used herein, means that the implant is constructed and arranged to deliver therapeutic levels of the imidazole of at least 30, and preferably 60 days. Such implants can be particularly useful in treating solid tumors by placing the implant near or directly within the tumor, thereby affecting localized, high-doses of the imidazole. Such implants can be especially useful in delivering imidazoles that are not successfully ingested, or that do not pass biological barriers, such as the blood/brain barrier. They also can be used to avoid undesirable canulation, such as when brain tumors are being treated. Long-term sustained release implants are well known to those of ordinary skill in the art and include some of the release systems described above.

Oral administration for many conditions will be preferred because of the convenience to the patient, although topical and localized sustained delivery may be even more desirable for certain treatment regimens.

-11-

The imidazoles, when used in vivo, are administered in therapeutically effective amounts. A therapeutically effective amount means that amount necessary to delay the onset of, inhibit the progression of or halt altogether the onset or progression of the particular condition being treated. Such amounts will depend, of course, on the particular condition being treated, the severity of the condition, and individual patient parameters including age, physical condition, size, weight and concurrent treatment. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. It is preferred generally that a maximum dose be used, that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or for virtually any other reasons.

Generally, daily oral doses of active compound will be from about 0.01 milligrams/kg per day to 1000 milligrams/kg per day. Small doses (0.01 - 1 mg) may be administered initially, followed by increasing doses up to about 1000 mg/kg per day. In the event that the anti-angiogenic response in a subject is insufficient at such doses, even higher doses (or effective higher doses by a different, more localized delivery route) may be employed to the extent patient tolerance permits. Multiple doses per day are contemplated to achieve appropriate systemic levels of compounds.

Cancers treatable according to the invention are nonprostate cancers that are solid tumors. Such cancers include: biliary tract cancer; brain cancer; breast cancer; cervical cancer; choriocarcinoma; colon cancer; endometrial cancer; esophageal cancer; gastric cancer; intraepithelial neoplasms; liver cancer; lung cancer; lymphomas;

-12-

neuroblastomas; oral cancer; ovarian cancer; pancreas cancer; rectal cancer; sarcomas; skin cancer; testicular cancer; thyroid cancer; and renal cancer.

As discussed above, proliferation of prostate cancer cells can be hormone (testosterone)-dependent; that is, proliferation of prostate cancer cells can be inhibited or arrested by eliminating the presence of testosterone. One prior art method for eliminating testosterone is by treatment with ketoconazole which blocks testosterone synthesis. Ketoconazole, however, was ineffective in inhibiting the growth of epidermal and vascular smooth muscle cells.

The imidazoles useful in this invention act by a different mechanism of action and are useful in inhibiting the growth of endothelial and/or vascular smooth muscle cells. Unlike ketoconazole, they, therefore, are useful in inhibiting or arresting the growth of solid tumors that do not depend upon the presence of hormone synthesis for proliferation or nonproliferation (i.e., nonhormone-dependent cancers.)

It will be understood by those of ordinary skill in the art that the imidazoles of the invention are also useful in treating prostate cancer patients who have been castrated. Such patients have no source of testosterone and, therefore, no longer have an indication calling for treatment with ketoconazole or any other imidazole according to the teachings of the prior art. If, however, such patients do not respond sufficiently as a result of castration, then the prostate cancer may be treated according to the methods of this invention.

The imidazoles useful in the invention may be delivered in the form of anti-cancer cocktails. An anti-cancer cocktail is a mixture of any one of the imidazoles useful with this invention with another anti-cancer drug and/or supplementary potentiating agent. The use of cocktails in the treatment of cancer is routine. In this embodiment, a common administration vehicle (e.g., pill, table, implant,

-13-

injectable solution, etc.) would contain both the imidazole useful in this invention and the anti-cancer drug and/or supplementary potentiating agent.

Anti-cancer drugs are well known and include:

Aminoglutethimide; Asparaginase; Bleomycin; Busulfan; Carboplatin; Carmustine (BCNU); Chlorambucil; Cisplatin (cis-DDP); Cyclophosphamide; Cytarabine HCl; Dacarbazine; Dactinomycin; Daunorubicin HCl; Doxorubicin HCl; Estramustine phosphate sodium; Etoposide (V16-213); Floxuridine; Fluorouracil (5-FU); Flutamide; Hydroxyurea (hydroxycarbamide); Ifosfamide; Interferon Alfa-2a, Alfa 2b; Leuprolide acetate (LHRH-releasing factor analogue); Lomustine (CCNU); Mechlorethamine HCl (nitrogen mustard); Melphalan; Mercaptopurine; Mesna; Methotrexate (MTX); Mitomycin; Mitotane (o. p'-DDD); Mitoxantrone HCl; Octreotide; Plicamycin; Procarbazine HCl; Streptozocin; Tamoxifen citrate; Thioguanine; Thiotepa; Vinblastine sulfate; Vincristine sulfate; Amsacrine (m-AMSA); Azacitidine; Erythropoietin; Hexamethylmelamine (HMM); Interleukin 2; Mitoguazone (methyl-GAG; methyl glyoxal bis-guanylhydrazone; MGBG); Pentostatin; Semustine (methyl-CCNU); Teniposide (VM-26) and Vindesine sulfate.

Supplementary potentiating agents likewise are well characterized and include: Tricyclic anti-depressant drugs (e.g., imipramine, desipramine, amitriptyline, clomipramine, trimipramine, doxepin, nortriptyline, protriptyline, amoxapine and maprotiline); non-tricyclic anti-depressant drugs (e.g., sertraline, trazodone and citalopram);  $\text{Ca}^{++}$  antagonists (e.g., verapamil, nifedipine, nitrendipine and caroverine); Calmodulin inhibitors (e.g., prenylamine, trifluoroperazine and clomipramine); Amphotericin (e.g., Tween 80 and perhexiline maleate); Triparanol analogues (e.g., tamoxifen); antiarrhythmic drugs (e.g., quinidine); antihypertensive drugs (e.g., reserpine); and Thiol depleters (e.g., buthionine and sulfoximine).



-14-

The imidazoles of the invention, when used in cocktails, are administered in therapeutically effective amounts. A therapeutically effective amount will be determined by the parameters discussed above; but, in any event, is that amount which establishes a level of the drug in the area of the tumor which is effective in inhibiting tumor growth.

#### EXAMPLES

##### Materials

Abbreviations: ChTX, Charybdotoxin; CLT, clotrimazole; ECZ, econazole; MCZ, miconazole; FCZ, fluconazole; METZ, metronidazole; IbTX, iberotoxin; KTX, kaliotoxin; DIDS, di-isothiocyano-disulfonyl stilbene; hemoglobin concentration; MCHC, mean corpuscular hemoglobin concentration; MOPS, 3-[N-morpholino]propanesulfonic acid.

##### Drugs and Chemicals

Synthetic charybdotoxin (ChTX) was purchased from Peptides International (Louisville, KY). A23187 was purchased from Calbiochem-Behring (LaJolla, CA). Fluconazole was provided by Pfizer Inc., Groton, CT, disulfonic acid (MOPS), clotrimazole (CLT), miconazole, econazole, metronidazole, and all other drugs and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and Fisher Scientific Co. (Fair Lawn, NJ), and the radioisotope  $^{86}\text{Rb}$  from Dupont (Billerica, MA).

##### Assays for Cell Proliferation

DNA synthesis assessed by the uptake of [3H]thymidine: Cells are grown in either 48 or 96 wells plates (Costar, Cambridge, MA) at  $10^4$  and  $0.8 \times 10^3$  cells per well, respectively, and grown in Dubelcco's modified Eagle's medium (DME, Gibco, Grand Island, NY) supplemented with 10% heat-inactivated calf serum; they are kept at 37°C in 5%  $\text{CO}_2$ . When they reach confluence, usually between 3 and 4

-15-

days, the medium is replaced with DME 0.5% serum to make them quiescent, and mitogenesis assays are performed 24 hours later.

Quiescent cells are exposed to a mitogenic stimulus, such as 10% serum, PDGF (Sigma Co. St. Louis, Mo), bFGF (Upstate Biotechnologies, Lake Placid, NY), or other appropriate mitogen according to the cell line, and 3 hours later 1  $\mu$ Ci/ml of [3H]thymidine (Dupont, Billerica, MA) is added to the wells, and the cells maintained at 37°C/5% CO<sub>2</sub> for additional 21 hours. Then the cells are washed 3 times with DME medium and the acid-precipitable radioactivity is extracted with cold 10% TCA (Sigma, Co). After neutralization with 0.3 N NaOH (Sigma Co.), aliquots are counted in a Packard Tri-Carb Scintillation counter (Packard Instrument, Downer's Grove, IL).

Measurement of cell density in culture plates: Cells of a specific test cell line are seeded at precisely the same low density in culture plates and incubated for approximately 12 hours in DME 10% serum, or other culture medium depending on the cell line tested. After 12 hours, the test drug, for example clotrimazole 10  $\mu$ M, is added to the cell medium of one plate and a similar amount of only the carrier of the drug, for example ethanol 10  $\mu$ l, to another plate. After 48 to 74 hours, the cell density in control (ethanol) and experimental (clotrimazole) plates is assessed under a light inverted microscope, by measuring the surface of the culture plate covered by the cell monolayer. Alternatively, the cells can be detached from the plate by incubation with trypsin (Sigma, Co.) 50% (v/v) in ethylene diaminetetraacetic acid (ECTA; Sigma, Co); then the cells are counted in an hemocytometer chamber (Fisher, Pittsburgh, PA).

#### Assays for Inhibitors of Ca<sup>++</sup> Activated K Channel

Ca<sup>++</sup>-sensitive K<sup>+</sup> channels have wide distribution among cells, including the human red cell where they were originally discovered and which is the most commonly utilized

-16-

assay system for activators and inhibitors of the channel for the following reasons: they are readily available, can be easily manipulated in the laboratory, and transport assays can be accurately standardized by reading the hemoglobin concentration of a red cell suspension.

**Preparation of Human Red Blood Cells:** Blood is collected in heparinized tubes and centrifuged in a Sorvall centrifuge (RB 5B, Du Pont Instruments, Newtown, CT) at 5°C for 10 minutes at 3000 g. Plasma and buffy coat are carefully removed and the cells washed four times with a washing solution containing 150 mM choline chloride (Sigma Co), 1mM MgCl<sub>2</sub> (Sigma Co), 10mM Tris-MOPS (Sigma, Ca), pH 7.4 at 4°C (CWS). An aliquot of cells is then suspended in an approximately equal volume of CWS, and from this original cell suspension hematocrit (Hct) and hemoglobin (optical density at 540 nm) are determined.

**Methods to Test Inhibitors of the Ca<sup>++</sup> Activated K channel:** To test inhibitors of the Ca<sup>++</sup> activated K channel, the channel is activated using the calcium ionophore A23187 (Calbiochem).

**By Atomic Absorption Spectrometry:** Washed human erythrocyte are suspended at an hematocrit = 1% in CWS containing 0.150mM CaCl<sub>2</sub> (Sigma Co). Aliquots of 1 ml are removed at 0, 3 and 5 minutes, layered on top of 0.3ml of the oil n-butyl phthalate (Fair Lane, NJ) placed in an Eppendorf microtube (Fisher) and then centrifuged in a micro centrifuge for 20 seconds. At time 5.30 minutes, ionophore A23187 (1μM final concentration) is added and samples removed and spin down through phthalate at times 6, 7, 8 and 9 minutes. The supernatant on top of the oil layer is removed and its K<sup>+</sup> concentration is measured by atomic absorption spectrometry using a Perkin Elmer model 5000 spectrometer (Perkin Elmer Corp., Norwalk, CT). The efflux of K<sup>+</sup> (mmol/l cells/h) in the absence and presence of the inhibitor is calculated from the slope of the curves relating the K<sup>+</sup> concentration in the supernatants (mmol/l cells) vs. time (min.).

-17-

By radioisotopic measurement of  $^{86}\text{Rb}$  influx. The incubation medium is the same but contains 2 mM KCl and 1  $\mu\text{Ci/ml}$  of the radioactive tracer  $^{86}\text{Rb}$ . After spinning the samples through the phthalate layer, the tubes are rapidly frozen ( $-80^\circ\text{C}$ ) by immersion in methanol-dry ice, the tips of the tubes containing the packed red cells cut, and counted in a Packard Gamma Counter.

#### Example 1

The inhibitory effect of clotrimazole (CLT) on cell proliferation was assessed in normal, non-cancerous cells.

Rat vascular smooth muscle cells (murine cell line): Quiescent cells were stimulated with purified growth factors (PDGF and bFGF, 5  $\mu\text{M}$ ) and synthesis of DNA was assessed by the incorporation of  $[^3\text{H}]$ thymidine measured 24 hours later. As shown in Fig. 1, 10  $\mu\text{M}$  CLT completely inhibited both PDGF and bFGF stimulated DNA synthesis. The effect was not due to a toxic, non-specific, effect because it was reversed by removing CLT and re-stimulating the cells with the corresponding growth factor (Fig. 1).

#### Example 2

Dose response inhibitors of DNA synthesis by clotrimazole was tested using rat vascular smooth muscle cells as described above. Clotrimazole was tested at concentrations of 0.001  $\mu\text{M}$ , 0.1  $\mu\text{M}$ , 1  $\mu\text{M}$  and 10  $\mu\text{M}$ . Cells were stimulated using 5  $\mu\text{M}$  bFGF. Inhibition was dose dependent, with 45% inhibition at 1  $\mu\text{M}$  and complete inhibition at 10  $\mu\text{M}$ . The  $\text{ID}_{50}$  was about 1.5  $\mu\text{M}$ . (Fig. 2)

#### Example 3

Bovine endothelial (BAEC) and human umbilical vein (HUVEC): Cells were seeded at a low density ( $2.5 \times 10^5$ ) in cell culture flasks (75 ml flasks) containing DME 10% calf serum (BAEC) or fetal calf serum (HUVEC); after 12 hs, when

-18-

the cells were attached to the surface of the flasks, CLT (10  $\mu$ M) or carrier (ethanol) were added to triplicate flasks. After 48 hs cell growth was assessed by optic microscopy calculating the surface of the culture flask covered by the cell monolayer. Both BAEC and HUVEC cells had covered  $90 \pm 2\%$  of the flask surface in the absence and less than  $10\%$  in the presence of CLT (data not shown).

#### Example 4

Other antimycotics were tested for their inhibition of bFGF-stimulated DNA synthesis in rat vascular smooth muscle cells. As shown in Figure 3, at a concentration of 10  $\mu$ M, 3 compounds, CLT, econazole (ECZ) and miconazole (MCZ) inhibited DNA synthesis. The order of inhibitory potency was CLT more potent than ECZ, and ECZ more potent than MCZ. In contrast, other inhibitors of the  $\text{Ca}^{++}$  activated K channel, namely Charybdotoxin, kaliotoxin and iberotoxin, also failed to inhibit DNA synthesis.

#### Example 5

The inhibitory effect of (CLT) on the  $\text{Ca}^{++}$  activated K channel of human erythrocytes was assessed in the presence of 60  $\mu$ mol A23187/L cell and 100  $\mu$ M  $\text{CaCl}_2$ . CLT markedly inhibited the  $\text{Ca}^{++}$  activated  $^{86}\text{Rb}$  influx and K efflux. Mean values of  $\text{ID}_{50}$  (calculated with Dixon plot analysis) was  $143 \pm 60$  nM (n=3).

Other antimycotics were tested for their inhibition of the  $\text{Ca}^{++}$  activated  $^{86}\text{Rb}$  influx human erythrocytes. The order of inhibitory potency was clotrimazole more than miconazole; and both of these were more than econazole. There was no inhibition by fluconazole, ornidazole and tinidazole, 2 related compounds, and only marginal with mitronidazole a member of the nitroimidazole group (Figure 4).

-19-

## Example 6

CLT inhibits the mitogenic activity released from endothelial cells by activated component. When endothelial cells (EC) in culture (both BAEC and HUVEC) are treated with terminal complement components to form the MAC (membrane attack complex of complement), they release into the culture medium a potent mitogenic activity that stimulates the proliferation of quiescent cells used as indicators of the mitogens. Both, quiescent Swiss 3T3 and vascular smooth muscle cells are stimulated by the mitogens released from EC in response to the MAC (Figure 5; Halperin et al. unpublished observation). Moreover, immunoprecipitation with specific antibodies has documented that both PDGF and bFGF released from the EC contribute in approximately equal proportion to the mitogenic activity induced by the MAC (data not shown).

To determine whether CLT inhibited the cell proliferative activity released by the MAC from EC, quiescent 3T3 and vascular smooth muscle cells were stimulated in the presence and absence of 10  $\mu$ M CLT with conditioned media obtained from MAC treated EC. The results indicate that CLT completely inhibited the proliferative response to mitogens released from EC (Figure 5).

Those skilled in the art will be able to ascertain with no more than routine experimentation numerous equivalents to the specific imidazoles and processes described herein. Such equivalents are considered to be within the scope of the invention and are intended to be embraced by the following claims in which we claim:

-20-

CLAIMS

1. A method for treating an angiogenic condition comprising:

administering to a subject in need of such treatment an imidazole that inhibits the  $\text{Ca}^{++}$  activated potassium channel of erythrocytes of the subject and that inhibits vascular smooth muscle cell proliferation.

2. A method as claimed in claim 1 wherein the imidazole is administered to a subject who has an angiogenic condition selected from the group consisting of: hemangioendotheliomas, hemangiomas and Kaposi's Sarcoma.

3. A method as claimed in claim 1 wherein the imidazole is administered to a subject who has diabetic retinopathy.

4. A method as claimed in claim 1 wherein the imidazole is administered to a subject who has psoriasis.

5. A method as claimed in claim 1 wherein the imidazole is administered to a subject who has prostate cancer and who has been castrated.

6. A method as claimed in claim 1 wherein the imidazole is administered to a subject who has a nonprostate solid tumor.

7. A method as claimed in claim 1 wherein the imidazole is administered to a subject who has a nonhormone dependent solid tumor.

8. A method as claimed in claim 1 wherein the imidazole is administered to a subject who has neovascular glaucoma.

-21-

9. A method as claimed in claim 1 wherein the imidazole is administered to a subject who has rheumatoid arthritis.

10. A method for treating an angiogenic condition, comprising:

administering to a subject in need of such treatment an imidazole that inhibits the  $\text{Ca}^{++}$  activated potassium channel of erythrocytes of the subject and that inhibits endothelial cell proliferation.

11. A method as claimed in claim 10 wherein the imidazole is administered to a subject who has an angiogenic condition selected from the group consisting of: hemangioendotheliomas, hemangiomas and Kaposi's Sarcoma.

12. A method as claimed in claim 10 wherein the imidazole is administered to a subject who has diabetic retinopathy.

13. A method as claimed in claim 10 wherein the imidazole is administered to a subject who has psoriasis.

14. A method as claimed in claim 10 wherein the imidazole is administered to a subject who has prostate cancer and who has been castrated.

15. A method as claimed in claim 10 wherein the imidazole is administered to a subject who has a nonprostate solid tumor.

16. A method as claimed in claim 10 wherein the imidazole is administered to a subject who has a nonhormone-dependent solid tumor.



-22-

17. A method as claimed in claim 10 wherein the imidazole is administered to a subject who has neovascular glaucoma.

18. A method as claimed in claim 10 wherein the imidazole is administered to a subject who has rheumatoid arthritis.

19. A claim as claimed in any one of claims 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, and 18, wherein the imidazole is selected from the group consisting of clotrimazole, miconazole and econazole.

1/3

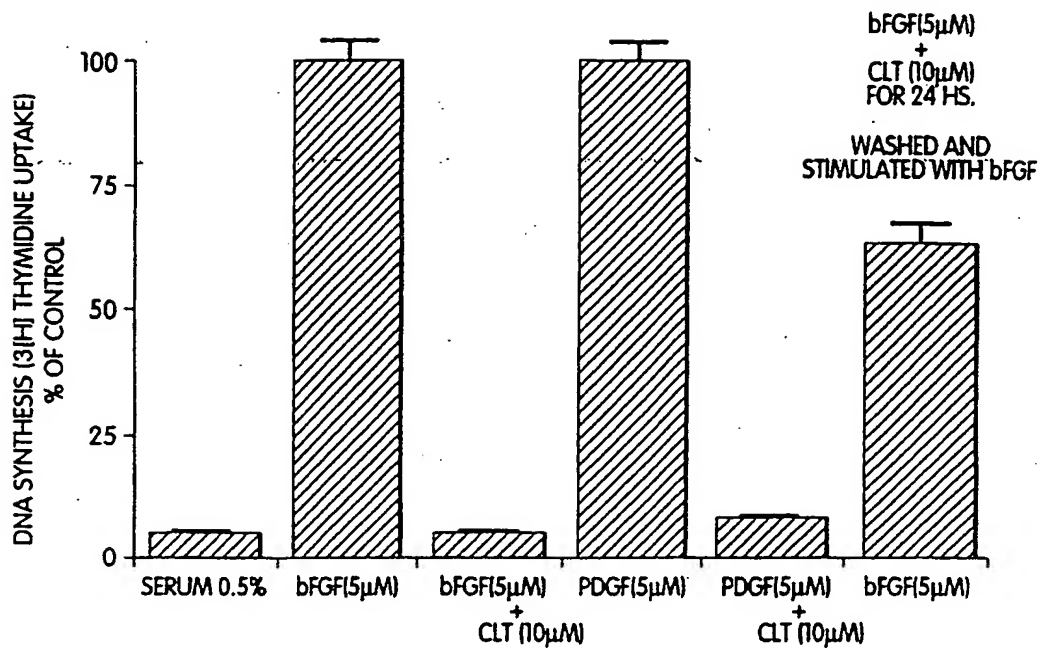


Fig. 1

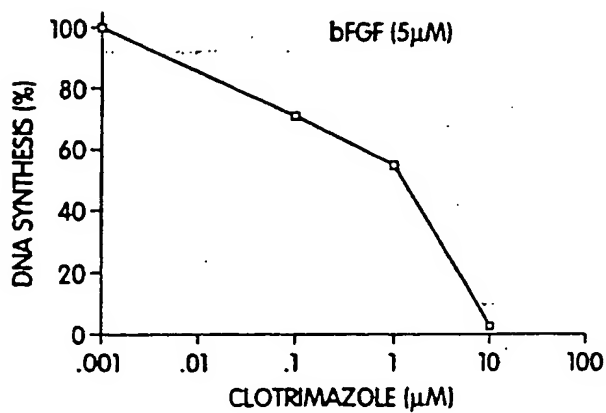


Fig. 2

SUBSTITUTE SHEET

2/3

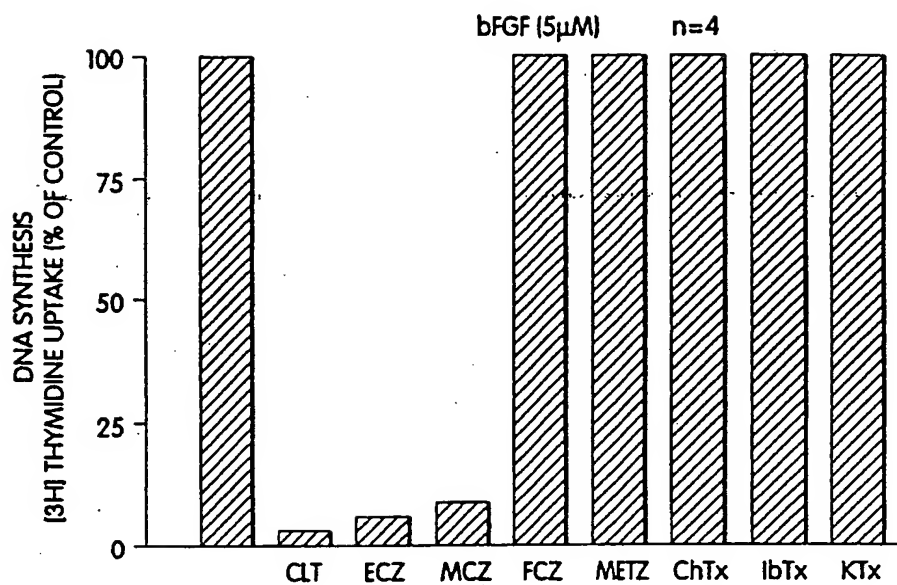


Fig. 3

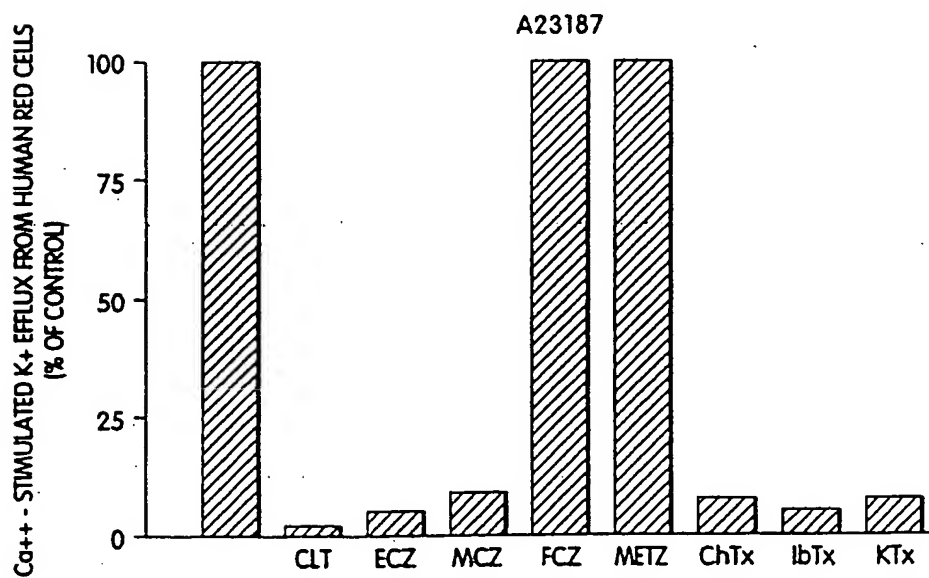


Fig. 4

SUBSTITUTE SHEET

3/3

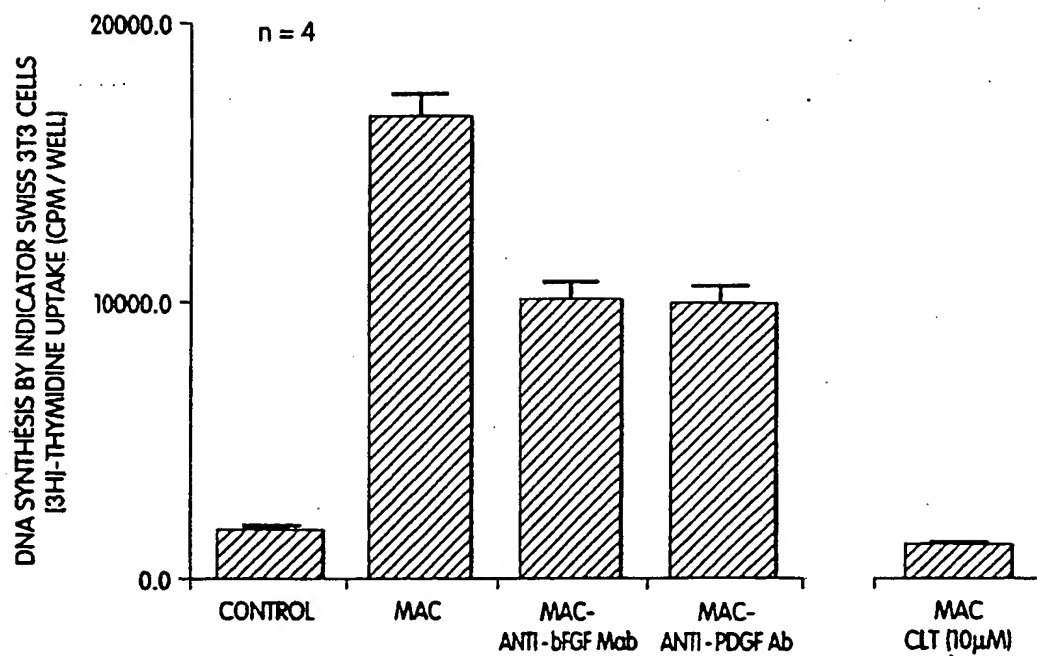


Fig. 5

SUBSTITUTE SHEET

## INTERNATIONAL SEARCH REPORT

Int. application No.  
PCT/US94/01740

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61K 31/415

US CL : 514/396, 399

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/396, 399

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US, A, 3,678,030 (Yamazaki et al) 18 July 1972, Abstract.	<u>1,2,10,11,19</u> 1,2,10,11,19
X --- Y	US, A, 3,901,908 (Fitzi et al) 26 August 1975, columns 1 and 2.	1, 9, 10, 18 ----- 19
X --- Y	US, A, 3,940,486 (Fitzi) 24 February 1976, see Abstract.	1,9,10,18 ----- 19
X --- Y	US, A, 4,491,588 (Rosenburg et al) 01 January 1985, see entire document.	1,4,10,13 ----- 19

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	T
"A" document defining the general state of the art which is not considered to be part of particular relevance	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than ... "A" ... the priority date claimed	document member of the same patent family ...

Date of the actual completion of the international search

17 MAY 1994

Date of mailing of the international search report

JUN 15 1994

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Authorized officer

BARBARA FRAZIER

Facsimile No. (703) 305-3230

Telephone No. (703) 308-1235

## INTERNATIONAL SEARCH REPORT

Int'l application No.  
PCT/US94/01740

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	US, A, 4,657,925 (Horn) 14 April 1987, columns 1-3, claims 1, 8, 12, 15.	1, 3, 8, 10, 12, 17 <hr/> 19
X — Y	US, A, 4,837,333 (Manley et al) 06 June 1989, columns 2 and 3.	1, 3, 8, 10, 12, 17 <hr/> 19
X — Y	US, A, 4,942,162 (Rosenberg et al) 17 July 1990, see entire document.	1,4,10,13 <hr/> 19
Y	US, A, 5,059,590 (Ueda et al) 22 October 1991, columns 1-3, Test Examples 1-3 at columns 16-18.	1, 9, 10, 18, 19

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/01740

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

(Telephone Practice)  
Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:  
1, 3, 4, 8-10, 12, 13, 17-19
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

Int. l. application No.  
PCT/US94/01740

### B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS ONLINE

search terms: hemangioma#, hemangioendothelioma#, sarcoma#, glaucoma#, retinopath?, psoriasis, dermatitis, dermatolog?, arthritis, rheumat?, angiogen###, imidazole#, clotrimazole, miconazole, econazole

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

- I. Claims 1, 2, 10, 11, and 19, drawn to treating hemangioendotheliomas, hemangiomas, and Kaposi's Sarcoma, classified in Class 514, Subclass 365.
- II. Claims 1, 3, 8, 10, 12, 17, and 19, drawn to treating diabetic retinopathy or neovascular glaucoma, classified in Class 514, Subclass 365.
- III. Claims 1, 4, 10, 13, and 19, drawn to treating psoriasis, classified in Class 514, Subclass 365.
- IV. Claims 1, 5, 10, 14, and 19, drawn to treating prostate cancer in a castrated subject, classified in Class 514, Subclass 365.
- V. Claims 1, 6, 7, 10, 15, 16, and 19, drawn to treating a nonprostate or a nonhormone dependent solid tumor, classified in Class 514, Subclass 365.
- VI. Claims 1, 9, 10, 18, and 19, drawn to treating rheumatoid arthritis, classified in Class 514, Subclass 365.

The six groups are directed to different inventions which are not linked as to form a single general inventive concept. The inventions are not linked in operation or effect, and arrive at completely different effects. Each group has a different purpose, that is, to treat a different disease or group of diseases. Each group of diseases has previously been treated by different methods, and thus it cannot be determined that each drug (i.e., each imidazole-containing compound) would be effective for each method of treatment recited.



**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

☒ **BLACK BORDERS**

☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**

☐ **FADED TEXT OR DRAWING**

☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**

☐ **SKEWED/SLANTED IMAGES**

☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**

☒ **GRAY SCALE DOCUMENTS**

☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**

☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**

☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**